IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	P-576 (TI-0022)
Inventors:	Huber et al.
Serial No.:	09/770,410
Filing Date:	January 25, 2001
Examiner:	Therkorn, Ernest G.
Customer No.:	26259
Group Art Unit:	1723
Confirmation No.:	6186
Title:	Method and Apparatus for Separating Polynucleotides Using Monolithic Capillary Columns

Electronically Submitted via EFS-Web

Date: September 5, 2006

I hereby certify that this paper is being electronically submitted on the date indicated above to the Commissioner for Patents, U.S. Patent & Trademark Office.

By _____
Typed Name: Jane Massey Licata, Reg. No. 32,257

Commissioner for Patents
U.S. Patent & Trademark Office

Dear Sir:

DECLARATION UNDER RULE § 1.131

We, Christian Huber, Herbert Oberacher and Andreas Premstaller, hereby declare that:

1. We are co-inventors in U.S. Patent Application Serial No. 09/770,410 filed June 7, 2000 and are most familiar with the subject matter of this application and the research effort which lead to the discovery of the instant invention. All the work described in the following paragraph occurred at the Institute of Analytical Chemistry and Radiochemistry in

Innsbruck, Austria, a recognized WTO member country since January 1, 1995.

2. We have reviewed Gusev et al. ((September 1999) *J. Chromatography* 855:273-290) and find that this reference describes a porous monolithic packing prepared with polystyrene-divinylbenzene support which is covalently attached to a fused silica capillary inner wall treated with a coupling agent trimethoxysilyl propyl methacrylate to provide anchoring sites for grafting of the polymer to the silica surface. The median pore radius for a monolithic sample prepared with ethanol is, as estimated by Gusev, about 5 micrometers.

3. Our invention referenced above, teaches a device for separating a mixture of polynucleotides by ion pair-reversed phase-high performance liquid chromatography. The device comprises a polymeric monolith having non-polar chromatographic surfaces. The monolith comprises an underivatized poly-(styrene/divinylbenzene) matrix and is contained within a tube having an inner diameter in the range of 1 to 1000 micrometers.

4. Laboratory protocol notebooks regarding experiments related to this invention were kept by Andreas Premstaller as a Ph.D. student under the direction of Christian Huber.

5. Andreas Premstaller worked in Christian Huber's laboratory during 1998 and 1999.

6. According to laboratory protocol notebooks submitted herewith, the first synthesis of PS/DVB monolith using decanol and tetrahydrofuran as porogens was performed on August 6, 1998. We then succeeded in a first separation of proteins (lysosome from beta-lactoglobulin B) in a PS/DVB monolithic column on August 25, 1998. See, e.g., the chromatograph at the bottom right-hand corner of the fourth laboratory notebook page.

The first successful separation of oligonucleotides on a PS/DVB monolith synthesized with decanol/THF as porogens was February 9, 1999.

7. We were able to fully practice our invention described in the above-referenced patent application prior to the date of the publication of the Gusev paper. A copy of the relevant laboratory notebook pages hereby accompanies my declaration.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

August 30, 2006 Christian Huber
Date Christian Huber, Ph.D.

Date Herbert Oberacher, Ph.D.

Date Andreas Premstaller, Ph.D.

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Date	Christian Huber, Ph.D.
30.8.2006	Herbert Oberacher
Date	Herbert Oberacher, Ph.D.
Date	Andreas Premstaller, Ph.D.

The first successful separation of oligonucleotides on a PS/DVB monolith synthesized with decanol/THF as porogens was February 10, 1999.

7. We were able to fully practice our invention described in the above-referenced patent application prior to the date of the publication of the Gusev paper. A copy of the relevant laboratory notebook pages hereby accompanies my declaration.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date Christian Huber, Ph.D.

Date Herbert Oberacher, Ph.D.

25.07.2006 
Date Andreas Premstaller, Ph.D.

5.8.98

C10OH sicher 2a... 28

Monolithensynthese mit THF M11

Nr.	Datum	Kapillare ID/OD [µm]	Polymerisationsmischung Styrol [ml]	DVB [ml]	AIBN [g]	C12OH [ml]	THF [ml]	Temperatur [°C]
M11_1	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	3.00	0.00	70, TS
M11_2	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.90	0.10	70, TS
M11_3	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.80	0.20	70, TS
M11_4	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.70	0.30	70, TS
M11_5	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.60	0.40	70, TS

THF sollte [illegible] als Hexan sein und [illegible] als Toluol

THF destilliert, da mit Radikalfänger (Phenol) stabilisiert.

Ausgangsmaterial: VS 3.8.98 trocken
THF dest.

Start: 6.8.98 10⁰⁰ h   T = 70°C

Ende: 7.8.98 12⁰⁰ h   T = 20°C

7.8.98

M11_1   15 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	1	0.05
10	1	0.06
25	4	0.25
50	7	0.44
100	14	0.86
150	21	1.31
200	28	1.75
k [bar cm⁻¹ µl⁻¹ min]		0.008712

M11_2   15 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	4	0.27
50	8	0.53
100	14	0.93
150	20	1.33
200	25	1.67
k [bar cm⁻¹ µl⁻¹ min]		0.008303

M11_3   15 cm   flow   constant-pressure

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	3	0.20
50	6	0.40
100	11	0.73
150	14	0.93
200	19	1.27
k [bar cm⁻¹ µl⁻¹ min]		0.006114

M11_4   16 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	9	0.56
10	14	0.88
25	32	2.00
50	68	4.25
100	126	7.88
150	180	11.25
k [bar cm⁻¹ µl⁻¹ min]		0.074602

M11_5   16 cm   200bar Gegendruck

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
1	21	1.31
2	33	2.06
3	49	3.08
4	63	3.94
5	77	4.81
7	92	5.75
10	170	10.63
12	184	11.50
k [bar cm⁻¹ µl⁻¹ min]		0.963149

Anteil THF [% Porogen]	Steigung k [bar cm⁻¹ µl⁻¹ min]
0.0%	0.00871
3.3%	0.00830
6.7%	0.00611
10.0%	0.07460
13.3%	0.96315

Mikroskop: 55 Skalenteile: 450 µ   1 Teil → 8 µm

M11_1 kleine Poren ~3µ
sehr porös.

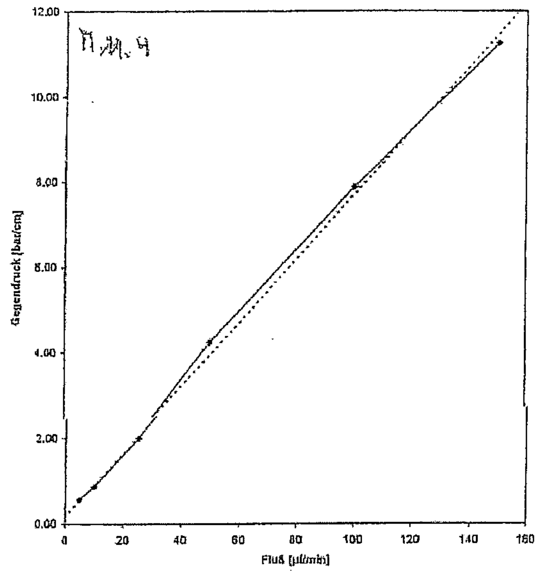
M11_2 große Poren, nicht gleichmäßig

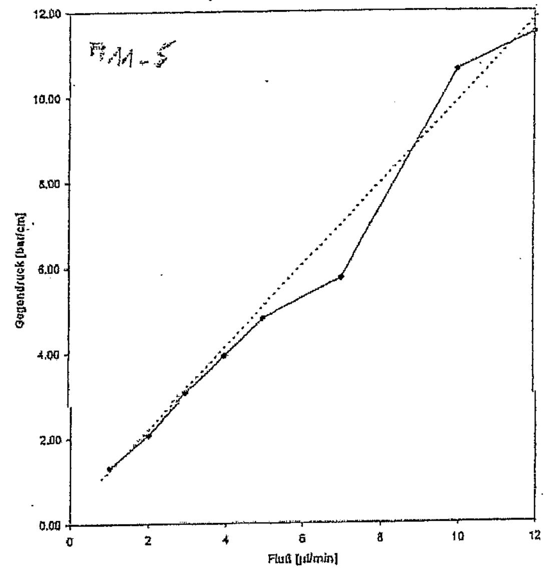
M11_3 große Poren, einzelne Kanäle [illegible]

M11_4 sehr dicht, keine Risse, porös, da nicht genau zu sehen

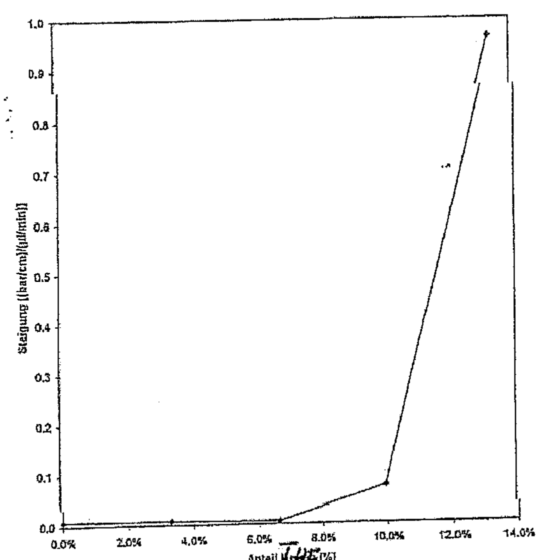
M11_5 keine Monolithstruktur zu erkennen.

ab





Abhängigkeit des Gegendrucks vom Anteil an THF im Porogengemisch



Als nächstes Bereich zwischen MM-4 und MM-5 genauer untersuchen.

Für HPLC MM-4 und MM-5 testen.

25.05.98

JM 11.5 vom 6.8.98

kein Spülen 240 bar / 5 µl/min

Sykam, 130 µl/min → Split → 4.6 µl/min

2 min 11 sec / 10 µl

2 min 30 sec / 4 µl/min

File. AP80875.SMP

GYNKOSOFT

Equilibrium:

(A) H_2O, 0.1% TFA

(B) ACN, 0.1% TFA

50% A, 14.50 –

Edelstahl-T-Stück statt Kapillare T-Stück

10 µl, 2 min 15 sec $\frac{10}{2.25}$ 4.44 µl/min

Bedingungen:

Thioharnstoff 0.05% in H_2O 50% ACN, 0.1% TFA

p ≈ 200 bar

100% H_2O, 0.1% TFA: Proteine zeigen keinen Peak → klebt an Kapillare?

Thioharnstoff nach ca. 1.50 min

50% ACN, 0.1% TFA: Proteine gleichzeitig mit Thioharnstoff: keine Retention

RIBA

27.8.98

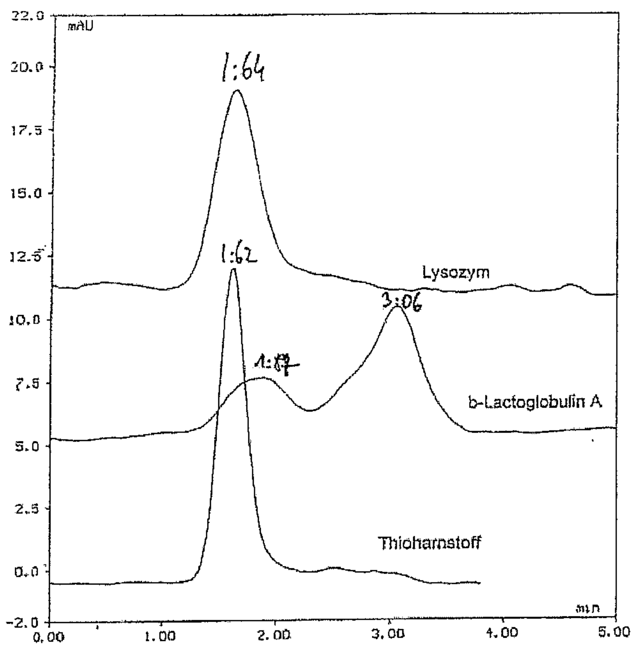
50% ACN, 0.1% TFA: Protein zurückgehalten, eluiert als Peak.

LAC A.

LYS keine Retention

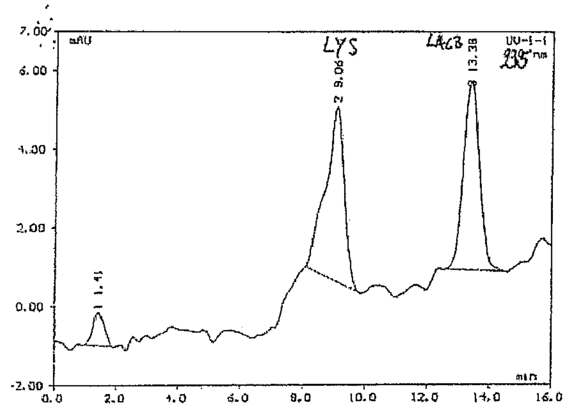
Nun 40% ACN

21



50% ACN, 0.1% TFA
inj 2mg/ml Protein
Retention von LacA bei 50% ACN

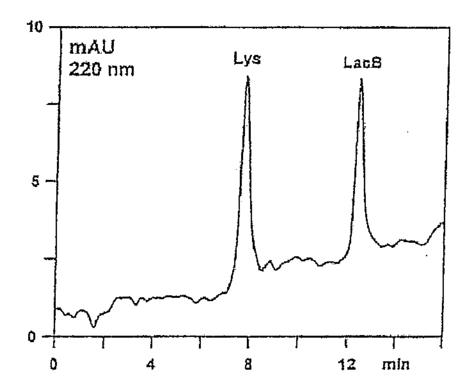
```
DP.Integration                SYS1 - C:A980825.SHP                Page  2
PSDVB 100x0.32mm, C12OH/THF-Porogen, M11_5 98086          1998-08-27/20:13
------------------------------------------------------------------------
Systaes 1mg/ml                      UV-1-1                    1998-08-27
Modified. H2O/30-60.15minACN/0.1%TFA,4.5/130ul/min,25°C    SynkoSoft V5.50
------------------------------------------------------------------------
Smp. No/Pos: 35/1             Control:                   Standard:  --------
Sample Type: Integration      Signals: ANDI1_SIG         Inject:    20.0 uL
Acquisition: 1998-08-27/19:53 Report:                    Dil. Fact.: 1.00000
Method:      DEFAULT.INT      P-Table:                   Weight:    1.00000
```



No.	Ret. Time min	Type	Area mAU*min	Height mAU	Half Width min	Base Width min	Plates
1	1.414	MB	3.351e-1	0.84	0.415	0.720	64
2	9.060	BMB	3.170e+0	4.46	0.627	0.972	1156
3	13.379	BMB	2.920e+0	4.32	0.567	0.988	3080
---	-------	---	6.445e+0	10.12	-------	-------	--------

Erfolgreiche Proteintrennung:
Lys, LACB je 1mg/ml, 20nl Inj.
30-60% ACN/15min, 0.1% TFA
4.5/130µl/min
215 nm

A980825-36



Separation of proteins in a monolithic capillary column

Column, PS-DVB (monolith, 100 x 0.32 mm); chromatographic conditions, mobile phase, (A) H_2O, 0.1% TFA, (B) ACN, 0.1% TFA, linear gradient, 30-60% B in 15 min; flow rate, 4.5 µl min^{-1}; temperature, 25 °C; detection, UV, 220 nm; sample, lysozyme, β-lactoglobuline B, 20 ng each.

OP 021 PPS

Versuch einer Trennung von Oligonucleotiden an Monolithen M13_5

M13-5

$l = 87\,mm$, $id = 200\,\mu m$

Eluenten: A: 50mM TEAA pH 6.8

B: 50mM TEAA 20% ACN pH 6.8

Temperatur: 50°C

Splitkapillare TSP075375, 6 cm Fluss 12 / 3.3 µl/min / 94 bar

File: A990209...STR

Trennung von dT_8, dT_{16}

Peakbreite mit Gradient 0–100% B/min. 0.11 min = 6.6 s

Trennung mit dT_{12-18}

Verschiedene Gradienten versucht.

gute Trennung: 30–50% B/10 min

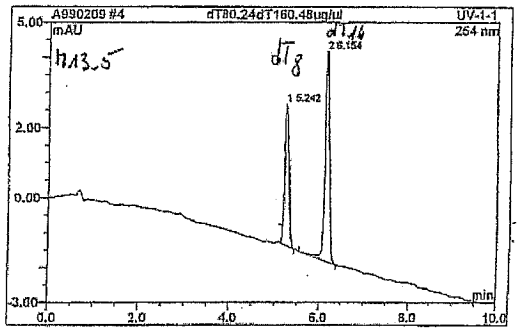
6 – 10% ACN/10 min

Operator:o72551 Timebase:A990209 Sequence:A990209 Page 4-1 10.2.1999 2:35 PM

4 dT80.24dT160.48µg/µl

0-100%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;0:2min;50°C

Sample Name: dT80.24dT160.48µg/µl Injection Volume: 20.0 nL
Control Program: Channel: UV-1-1
Quantit. Method: OLIGO1 Recording Time: 09.02.99 10:00



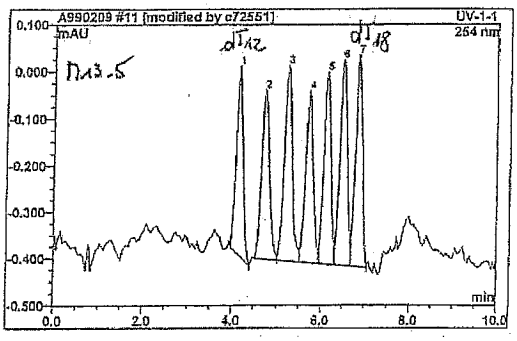
No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	5.242	0.450	4.053	0.109	13593	1.303
2	6.154	0.744	6.008	0.111	16926	1.331
Total:		1.194	10.061			

Operator:o72551 Timebase:A990209 Sequence:A990209 Page 11-1 10.2.1999 2:34 PM

11 dT12-18 0.25µg/µl

30-50%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;0:2min;50°C

Sample Name: dT12-18 0.25µg/µl Injection Volume: 20.0 nL
Control Program: Channel: UV-1-1
Quantit. Method: OLIGO1 Recording Time: 09.02.99 21:29



No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	4.155	0.075	0.405	0.175	3142	1.050
2	4.707	0.071	0.364	0.178	3899	1.551
3	5.224	0.068	0.420	0.162	4101	1.245
4	5.709	0.073	0.370	0.160	5552	1.084
5	6.122	0.062	0.412	0.189	5813	n.a.
6	6.463	0.066	0.441	0.182	7042	n.a.
7	6.835	0.062	0.454	0.171	8886	n.a.
Total:		0.556	2.866			